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Prolactin Gene Expression in Human Myometrial Smooth Muscle Cells Is Induced by Cyclic Adenosine 3',5'-Monophosphate

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We have previously characterized PRL production in human myometrial tissue maintained in explant culture. Here we describe PRL gene expression and its regulation in smooth muscle cells isolated from normal human myometrium. Onset of PRL secretion occurred spontaneously after several days in culture and increased over time without exogenous stimulation. PRL secretion could be further stimulated by the addition of PGE_2 or relaxin, both of which were also shown to increase cAMP formation in smooth muscle cells. Likewise, treatment with 8-Br-cAMP led to an elevation of PRL secretion. By reverse transcription/polymerase chain reaction, we demonstrate that smooth muscle cells transcribe the PRL gene from the alternative decidual-type dPRL promoter, located upstream of the pituitary promoter. Treatment with PGE_2, relaxin, and 8-Br-cAMP resulted in an increase in dPRL transcript abundancy. The effect of cAMP was transcriptional as shown by the induction of transfected dPRL promoter/reporter gene fusion constructs. A fragment of 332 bp flanking the dPRL transcription start site was sufficient to mediate cAMP inducibility. In parallel with the increase in PRL secretion, we detected an increase in cAMP formation and PGE_2 secretion in cultured smooth muscle cells. We propose the presence of a paracrine positive feedback mechanism that may reflect the physiological situation in vivo where an increase in myometrial adenylate cyclase activity throughout pregnancy has been reported.

Key Words: PRL; human; dPRL promoter; myometrium; smooth muscle cell; cAMP.

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Introduction

In the nonpregnant human uterus, decidualized endometrial stromal cells and the myometrium have been identified as sites of PRL production (Daly et al., 1983; Walters et al., 1983). Explant cultures from leiomyomas (fibroids), a benign pathologic process of uterine smooth muscle cells, have been reported to secrete higher amounts of PRL compared to normal myometrial explants (Rein et al., 1990), and the proliferation of SMC in monolayer culture is stimulated by added PRL (Környei et al., 1993). The hormone has therefore been implicated as a paracrine growth factor for fibroids. However, the regulation of myometrial PRL production remains poorly defined compared to that of endometrial stromal cells. Although cultured endometrial stromal cells remain PRL-negative unless provided with a long-term progestin treatment or a stimulus of adenylate cyclase activity (Huang et al., 1987; Irwin et al., 1989; Tabanelli et al., 1992; Tang and Gurpides 1993; Frank et al., 1994), myometrial and fibroid explants spontaneously begin to secrete increasing amounts of PRL after several days in culture (Daly et al., 1984; Gellersen et al., 1991; Stewart et al., 1994, 1995). Whereas PRL production from such explant cultures is inhibited by progestins (Walters et al., 1983; Daly et al., 1984; Gellersen et al., 1991), this effect could not be reproduced in purified monolayer cultures of SMC (Nowak et al., 1993) and may therefore be mediated through other cell types present in tissue explants. In addition, a lack of effect of estradiol, TRH, and IGF-I on PRL secretion was demonstrated in SMC cultures from fibroids, whereas insulin proved to be inhibitory (Nowak et al., 1993).

Gonadotropins and free α-subunit stimulate PRL secretion from myometrial explants (Stewart et al., 1994, 1995). The presence of LH/hCG receptors has been demonstrated on SMC, and purified SMC in culture respond to LH or hCG with an increase in cell density (Reshet et al., 1990; Környei et al., 1993), but a potential role of gonadotropins in regulation of PRL production has not been investigated.

PRL gene expression in human uterine tissues is initiated at an alternative upstream promoter leading to the formation of an elongated decidual-type PRL (dPRL) transcript (DiMattia et al., 1990; Gellersen et al., 1991; Hiraoka et al., 1991). It has become increasingly evident that dPRL gene transcription in endometrial stromal cells is regulated by the protein kinase A (PKA) pathway. Gonadotropins, PGE_2, and relaxin, which act to elevate intracellular cAMP and are locally present in vivo, as well as stable cAMP analogs, induce dPRL gene expression in endometrial stromal cells (Huang et al., 1987; Zhu et al., 1990; Tang et al., 1993; Tang and Gurpide, 1993; Frank et al., 1994). Since myometrial SMC and endometrial stromal cells are exposed to a similar hormonal environment in vivo, share a common embryological origin, and use the same alternative promoter of the hPRL gene, we decided to investigate the possible significance of cAMP in the control of myometrial PRL expression.

Results

The dispersed myometrial cells adhered readily to the culture dishes, despite the harshness of the digestion procedure, and reached confluency after 8–10 d. Virtually all cells stained positive for α-smooth muscle actin and vimentin. About 10–15% of the cells were positive for desmin, whereas cytokeratin staining was completely absent. The cultures therefore represented highly purified SMC. Variability of desmin expression in cultured SMC, depending on culture conditions, has been reported previously (Nowak et al., 1993). To characterize the secretory capacity of the cells, conditioned media and cell extracts were collected every 48 h for determination of PRL concentration and DNA content. Spontaneous secretion of PRL started around days 4–10, increased with time, and continued for more than 40 d. A representative time-course is shown in Fig. 1. In parallel, an increase in cAMP formation with time in culture was observed. Between days 7 and 13, the cAMP production rate, normalized to DNA content of the wells, increased more than twofold (Fig. 2).

We therefore tested whether ligands whose receptors are coupled to the adenylate cyclase system would affect PRL production from SMC cultures. Treatment of confluent cultures with PGE_2 ($10^{-6}M$) and relaxin (100 ng/mL), but not with hCG (100 ng/mL), resulted in a 7.6- and 3.6-fold stimulation of cAMP release after 45 min, respectively (Fig. 3). Treatment was then continued for 2 × 48 h, and the supernatant of the final 48-h incubation period was assayed for PRL. In accordance with the effect on cAMP formation, PGE_2 and relaxin, but not hCG, elicited a significant stimulation of PRL secretion (2.85- and 2.05-fold of untreated controls, respectively) (Fig. 3).

In the search for an autocrine signal that might account for the induction of PRL production, we assayed for PGE_2 in SMC-conditioned media. We were prompted to do so by

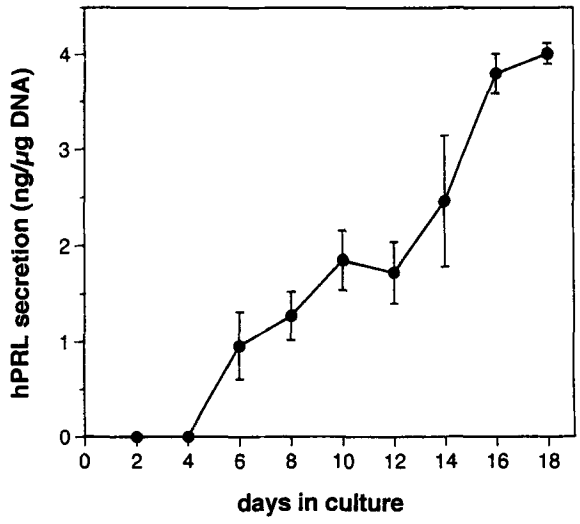

Fig. 1. Spontaneous PRL secretion by SMC in long-term culture. Cultures were initiated at 10^5 cells/well, and media were replaced every 48 h. Triplicate wells were used every 48 h to harvest conditioned media and DNA. PRL concentrations in the supernatants were normalized to DNA content of the respective wells.

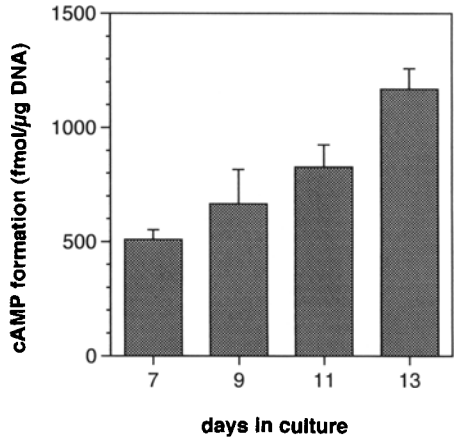

Fig. 2. Increasing cAMP release by SMC over time in culture. Six parallel wells were used per time-point to measure cAMP release in a 45-min incubation. The cAMP concentration in the supernatants was normalized to the DNA content of the respective wells. cAMP release at day 13 was significantly different from day 7 ($p < 0.01$) and day 11 ($p < 0.05$).

our initial observation that preparing SMC cultures in the presence of indomethacin, an inhibitor of prostaglandin biosynthesis, delayed the appearance of basal PRL production. As shown in Table 1, there is in fact a dramatic increase in PGE_2 secretion between days 11 and 15 in culture, coinciding with the secondary increase in PRL secretion.

To test whether the stimulation of PRL secretion from SMC occurred at the level of PRL gene expression, we performed RT-PCR analysis on cultures treated with PGE_2, relaxin, and 8-Br-cAMP. For the amplification of PRL cDNA, we used an upstream primer located in the 5' untranslated sequence specific for the dPRL transcript

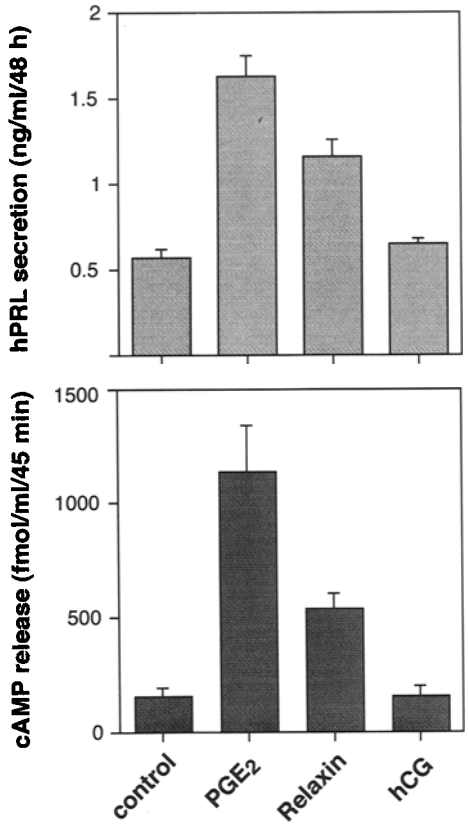

Fig. 3. Correlation between stimulation of cAMP release and PRL secretion. Triplicate cultures of confluent SMC were treated with PGE_2 ($10^{-6}M$), relaxin (100 ng/mL), or hCG (100 ng/mL). Supernatants were removed for cAMP assay after 45 min (lower panel) and for PRL assay after the second of two 48 h treatment periods (upper panel). PGE_2 and relaxin caused significant increases in hPRL secretion and cAMP release ($p < 0.001$).

Table 1
Coinciding Increases in PRL and PGE_2 Secretion from Cultured SMC

Days in culture	hPRL secretion, ng/μg DNA	PGE_2 secretion, ng/μg DNA
3	nd[a]	12.51 ± 2.33[b]
5	nd	10.23 ± 4.20
7	nd	9.22 ± 2.62
9	1.07 ± 0.03	7.62 ± 0.40
11	0.92 ± 0.03	8.09 ± 3.87
13	1.31 ± 0.06	27.16 ± 0.15
15	2.89 ± 1.09	69.74 ± 20.96

[a]Not detectable.
[b]Mean ± SD of triplicate cultures.

(Gellersen et al., 1994). Both PGE_2 and 8-Br-cAMP (0.5 mM) caused a strong induction of dPRL gene expression. A lower dose of 8-Br-cAMP (0.2 mM) and relaxin gave a less pronounced, but still detectable increase in dPRL mRNA abundance (Fig. 4). The level of induction obtained with 8-Br-cAMP was sufficient to render the dPRL transcript

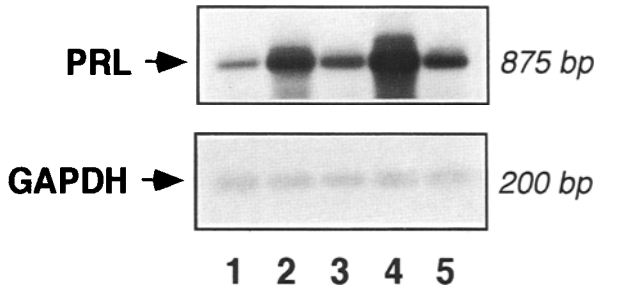

Fig. 4. RT-PCR analysis of PRL gene expression in SMC. Cells were left untreated (lane 1) or treated for 3 × 48 h with PGE_2 ($10^{-6}M$; lane 2), 8-Br-cAMP (0.2 mM; lane 3), 8-Br-cAMP (0.5 mM; lane 4), or relaxin (100 ng/mL; lane 5). RNA was reverse-transcribed and subjected to simultaneous PCR amplification using primers specific for dPRL and GAPDH cDNA sequences. The Southern blot of PCR products was hybridized with internal oligonucleotides.

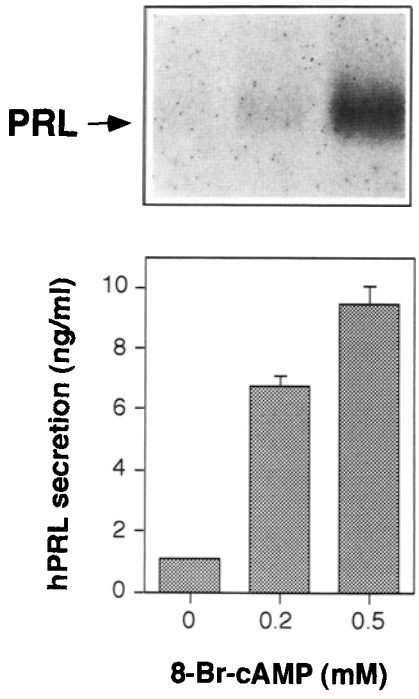

Fig. 5. Northern blot analysis of PRL mRNA in SMC. Quadruplicate cultures were treated with the indicated doses of 8-Br-cAMP for 3 × 48 h. Conditioned media were collected from the final 48-h period, and RNA was extracted. The upper panel shows a Northern blot of 22 μg RNA, hybridized with hPRL cDNA. Transcript size is approx 1.15 kb. The lower panel depicts PRL secretion from the corresponding cultures.

readily detectable by Northern blot analysis. The dose dependency of induction was apparent both at the level of dPRL mRNA abundance and PRL secretion (Fig. 5).

The effect of 8-Br-cAMP on the dPRL promoter was confirmed by transient transfection analysis. A construct carrying 3000 bp of DNA 5' to the decidual-specific transcription start site of the PRL gene, linked to the hGH reporter gene (dPRL-3000/GH), was inactive in untreated

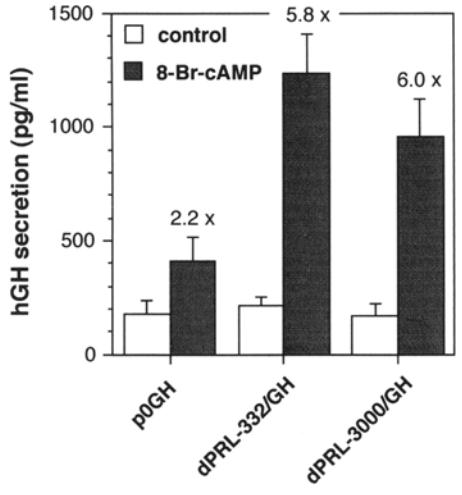

Fig. 6. Transient transfection analysis of dPRL promoter constructs in SMC. Triplicate wells were transfected with the promoterless hGH reporter gene construct pOGH, or with fusion constructs carrying 3000 or 332 bp 5'-flanking DNA to the dPRL promoter (dPRL-3000/GH and dPRL332/GH), in the absence (open bars) or presence (filled bars) of 8-Br-cAMP (0.5 m*M*). Conditioned media were collected 72 h later for hGH measurement. Numbers on top of columns indicate the fold inductions of a reporter gene construct in the presence of 8-Br-cAMP compared to the same construct in the absence of 8-Br-cAMP.

SMC, but clearly induced (5.8-fold) by the addition of 8-Br-cAMP, above the nonspecific twofold stimulation of the promoterless control construct. The same level of induction was obtained with a construct truncated to 332 bp 5' to the transcription start site (dPRL-332/GH) (Fig. 6).

Discussion

PRL production has previously been described in monolayer cultures of SMC derived from leiomyomas (Nowak et al., 1993). Here we show that SMC isolated from normal myometrial tissue also have the capacity to produce PRL. After several days in culture, the cells begin to secrete PRL. The spontaneous onset of PRL expression in SMC is in contrast to the hormone dependency of PRL induction in endometrial stromal cells, and also differentiates SMC from fibroblasts isolated from term decidua, which require stimulation with PGE_2 or a cAMP analog in combination with steroids to activate the PRL gene (Richards et al., 1995). We demonstrate here that the PRL gene in myometrial SMC, as in other uterine cell types, is responsive to stimulation of the PKA pathway. Treatment with PGE_2 or relaxin, leading to an elevation of cAMP formation, and exposure to exogenous 8-Br-cAMP, resulted in an increase in PRL secretion and PRL mRNA abundance. The stimulation occurred, at least in part, at the transcriptional level, since transfected fusion constructs carrying the dPRL promoter were also induced by 8-Br-cAMP.

A physiological ligand to which the myometrium is exposed in high concentrations during pregnancy is hCG. LH/hCG receptors have been identified on cultured SMC, and the cultures show an increase in cell density after 4 d of treatment with hCG (Környei et al., 1993). Myometrial explant cultures respond to hCG with an increase in PRL secretion (Stewart et al., 1995). However, in our monolayer cultures of myometrial SMC, hCG failed to exert an effect on either cAMP formation or PRL secretion. The reason for this is not clear, but it might be explained by the fact that we used tissue from premenopausal women, whereas in the study on explant cultures mentioned above, tissue from postmenopausal women proved to be far more responsive to hCG with respect to PRL secretion than that from premenopausal subjects (Stewart et al., 1995). Also, cellular responses are certainly variable depending on whether the cells are maintained in a tissue context in explants or as purified populations in monolayer culture. For example, the inhibition of PRL secretion from myometrial and fibroid explants by progestins is not seen in purified SMC cultures (Walters et al., 1983, Daly et al., 1984; Gellersen et al., 1991, Nowak et al., 1993).

The spontaneous initiation of PRL production in cultured SMC might be explained by the release from an inhibitor with time in culture and/or by the evolvement of a positive autocrine feedback loop. Our observations provide evidence for the latter mechanism, but do not exclude the first. Coinciding with the appearance of detectable PRL in the culture supernatants, the cells also began to release increasing amounts of cAMP and PGE_2. At the present stage, we cannot unravel the sequence of events or decide whether they are coincidental or causally linked. We have demonstrated previously that the dPRL promoter is cAMP-inducible in endometrial stromal cells (Gellersen et al., 1994), and this also applies to SMC as shown in this study. It is therefore conceivable that intracellular cAMP begins to rise in cultured SMC and induces the dPRL promoter. Cyclic AMP has also been shown to stimulate PGE_2 production, at least in chorion and decidua (Mitchell et al., 1994). PGE_2 in turn elevates intracellular cAMP, as has been demonstrated in endometrial stromal cells (Yee and Kennedy, 1993) and in this study. Such autocrine feedback between cAMP and PGE_2 may thus contribute to activation of the dPRL gene. It seems less likely that PRL is the trigger of events, since in vitro studies on endometrial explants failed to demonstrate a direct action of PRL on PGE_2 release (Salamonsen and Findlay, 1990).

The increase in intracellular cAMP that we observed in cultured SMC, and the possible involvement of PGE_2 in a paracrine control mechanism, have a correlate in vivo. Basal adenylate cyclase activity and the expression of the stimulatory G-protein subunit, $G_{\alpha S}$, have been reported to increase in the myometrium throughout pregnancy until term (Europe-Finner et al., 1993; Miyazaki et al., 1995). The main source of PGE_2 in pregnancy is the amnion (Kelly et al., 1992). Since PGE_2 couples to adenylate cyclase activity and increased cAMP leads to myometrial relax-

ation this mechanism may serve to maintain a quiescent uterus during gestation (Europe-Finner et al., 1993, 1994; Tanaka et al., 1993). Whether myometrial PRL expression, which is very low in the nonpregnant uterus (Gellersen et al., 1991), also increases during pregnancy remains to be established.

Materials and Methods

Cell Isolation and Culture

Uterine tissue was obtained within 2 h of surgery from premenopausal women undergoing hysterectomy for leiomyomas. The tissue was collected in Earle's buffered saline containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Gibco BRL, Eggenstein, Germany). Myometrial tissue was excised from the interior of normal myometrium, minced thoroughly, and washed twice in buffered saline. The fragments were incubated in Ham's F-12 (ICN, Meckenheim, Germany) containing 1 mg/mL collagenase (Serve, Heidelberg, Germany) and 2.5 mg/mL DNase I (Boehringer, Heidelberg, Germany) at 37°C for 16 h to isolate SMC (Casey et al., 1984). At the end of the incubation period, the suspension was filtered through a sterile nylon stocking and subsequently treated as previously described for endometrial stromal cell preparation (Gellersen et al., 1994). Cell viability, determined by trypan blue dye exclusion prior to plating, was approx 10%. Plating media was DMEM/Ham's F-12 (1:1) supplemented with 10% FCS, 17-β-estradiol ($10^{-9}M$), 100 U/mL penicillin, and 100 μg/mL streptomycin. The suspension was plated at a density of 1×10^5 cells/mL in 12- or 24-well plates. Medium was initially changed after 24 h to remove dead cells and debris, and then every 48 h.

Studies on the release of hPRL or PGE_2 in long-term cultures or on the effect of PGE_2, hCG, 8-Br-cAMP (Sigma, Deisenhofen, Germany), or porcine relaxin (NIH-RXN-P1; NIAMDD, Bethesda, MD) on hPRL production were performed in plating media in three to six parallel wells on at least three individual cell preparations. For measurement of cAMP formation, cells were washed and incubated in serum-free medium (Opti-MEM; Gibco) for 45 min. At the end of the experiments, supernatants were collected for assays of PRL (IRMA; intra- and interassay coefficients of variation <3.8 and 2.9%, respectively, over the range of the standard curve) (IBL, Hamburg, Germany), PGE_2 (competitive enzyme immunoassay; sensitivity >5 pg/mL) (Boehringer) or cAMP (radioimmunoassay on acetylated samples; intra- and interassay coefficients of variation <7.3% and 12.4%, respectively, over the range of the standard curve) (IBL). Cells were harvested for DNA quantification by fluorometry (Hoefer Scientific Instruments, San Francisco, CA) or for RNA extraction (RNAClean; AGS, Heidelberg, Germany). Statistical comparisons were performed by analysis of variance and Bonferroni correction.

Immunocytochemistry

Purity of the cell preparations was verified by immunocytochemistry. Primary cultures were grown on LabTek chamber slides (Nunc, Wiesbaden, Germany) and fixed with 2% paraformaldehyde. After permeabilization with Triton X-100 (Bio-Rad, Munich, Germany), they were processed for indirect immunofluorescence using the following primary monoclonal antibodies (MAbs): a 1:400 dilution of anti-α-smooth muscle actin (clone 1A4; Sigma, Deisenhofen, Germany), antihuman desmin (1 :25) (DAKO, Hamburg, Germany), antihuman cytokeratin (1 :50) (MN 116; DAKO), or a 1:2 dilution of antivimentin (V3260; Dianova, Hamburg, Germany) for 1 h at room temperature. As negative control a nonbiological hapten of the equivalent IgG_{2a} class (1:5) (Dianova) was used as well as the omission of the first or second antibody during the staining procedure. The secondary antibody was Cy3™-conjugated AffiniPure $F(ab')_2$ fragment goat antimouse IgG (Dianova), used at a 1:50 dilution for 30 min at room temperature, and visualized by fluorescence microscopy.

RNA Analysis

Northern Blot analysis was performed as previously described (Gellersen et al., 1991) using a 564 bp *PstI* fragment of the hPRL cDNA as a probe (Cooke et al., 1981). Complementary DNA was synthesized from 5 μg total RNA with SuperScript RNase H– reverse transcriptase (Gibco), and PCR was performed with *Pfu* polymerase (Stratagene, Heidelberg, Germany) as detailed previously (Gellersen et al., 1994). Simultaneous amplification of hPRL and GAPDH cDNAs was performed by adding 10 pmol each of the following oligonucleotides to one reaction: dPRL-5' (corresponding to positions –136 to –107 in the decidual-specific 5'-untranslated region [UTR] of the hPRL cDNA) (DiMattia et al., 1990), hPRL-3' (antisense to positions 710–739 in the 3'-UTR of the hPRL cDNA), GAPDH-5' (positions 363–380 in the GAPDH cDNA) (Tokunaga et al., 1987), and GAPDH-3' (antisense to positions 543–560). Southern blots of the PCR products were hybridized with biotinylated oligonucleotides dPRL-int (antisense to positions –115 to –96 in the decidual-specific 5'-UTR of the hPRL cDNA) and GAPDH-int (positions 454–472 in the human GAPDH cDNA) followed by detection with the Southern-Light chemiluminescent system (Tropix, Bedford, MS).

Transient Transfections

The generation of fusion gene constructs using hGH as the reporter gene has been detailed previously (Gellersen et al., 1994). Either 3000 or 332 bp of 5'-flanking DNA to the decidual-specific PRL promoter were inserted into the promoterless plasmid p0GH (Nichols Institute, Bad Nauheim, Germany) (Selden et al., 1986) to yield dPRL-3000/GH and dPRL-332/GH, respectively. Primary cultures of SMC were trypsinized and plated at 0.7×10^5 cells/well in

12-well plates the day before transfection. Plasmid DNA (2 μg) was mixed with 3.2 μL LipofectAmine (Gibco) in serum-free media (Opti-MEM) and applied to the cultures for 6 h. Cells were then washed and received fresh plating media with or without 8-Br-cAMP (0.5 m*M*). Supernatants were collected 72 h later for measurement of hGH concentrations by a modified chemiluminescence immunometric assay (hGH LumaTag; Nichols Institute) (Gellersen et al., 1994).

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